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BRIEF COMMUNICATION

Developmental expression of β-glucosidase in olive leaves

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Abstract

Plant β-glucosidases catalyze the hydrolysis of glycosidic linkages and play a vital role in defense against pathogens and stress. The present work investigated the relationship between leaf development and β-glucosidase protein content in *Olea europea* L. (cv. Picual) leaves. The total chlorophyll content increased with leaf age in current-season leaves. Immunoblot analysis revealed that the content of 61 kD protein of β-glucosidase also increased with leaf age, and that the enzyme existed in three isoforms (pI 5.8 - 6.2). Statistical analysis indicated a strong correlation between chlorophyll and β-glucosidase protein contents.

Additional key words: chlorophyll content, immunoblot, leaf development, *Olea europaea* L.

Plant β-glucosidases (EC 3.2.1.21) catalyze the hydrolysis of glycosidic linkages and play important roles in a variety of fundamental biological processes. Particularly, the β-glucosidase/β-glycoside system is one of the major chemical weapons plants use to defend themselves against pests and stress (Konno *et al*. 1999). β-Glucosidic substrates and β-glucosidases are stored in different subcellular or tissue compartments (Poulton 1990). For example, leaves of privet (*Ligustrum obtusifolium*) contain a large amount of oleuropein, a phenolic secoiridoid glycoside, which is stably kept in a compartment separating from activating enzymes. When the leaf tissue is destroyed by herbivores, specific β-glucosidase in organelles activates oleuropein into a very strong protein denaturant that has protein-cross-linking and lysine-decreasing activities (Konno *et al*. 1999). Similar β-glycosidase/oleuropein system can be expected in olive tree, which is within the same family (*Oleaceae*) as privet tree. At present little information about olive β-glycosidase is available. Only a cDNA sequence in GeneBank (Accession no. AY083162.1) is attributed to olive β-glucosidase, and β-glucosidase was found to be responsible for the removal of the bitter-tasting oleuropein in table olive fruit and oil (Ciafardini and Zullo 2002).

Olive cultivation is spread in many regions around the world with temperate and subtropical climate, constituting an economically important crop for most Mediterranean countries (Melgar *et al.* 2008). From the mid-term of last century on, olive trees have been introduced and planted in China for oil production. Olive tree retains its leaves for 2 - 3 years before abscission. The leaves are recalcitrant to common protein extraction methods due to high contents of interfering compounds. Especially, extraction of olive leaf protein for two-dimensional (2D) electrophoresis analysis is challenging. Recently, we have developed a protocol of protein extraction from olive leaves and other recalcitrant plant tissues (Wang *et al.* 2003, 2006) for electrophoretic analysis, which provides a good basis for characterization and purification of olive β-glucosidase. In the present study, we characterized the expression of β-glucosidase in olive leaves using immunoblot analysis with a specific polyclonal antibody, and examined the relationship between β-glucosidase protein content and leaf development.

Olive (*Olea europea* L. cv. Picual) leaf development was followed by measuring chlorophyll content from young to adult leaves in a current-season branch from an olive tree grown in natural conditions during summer

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Abbreviations: 2D - two-dimensional; pI - isoelectric point.
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2006. Leaves were numbered starting from the apex of the branch. The youngest leaf with a mid-rib length of 2.5 cm was defined as the first leaf. Leaf 5 was fully expanded with a mid-rib length of approximately 3.9 cm. Leaves Nos. 1 to 5 were collected and cut into two halves along the mid-rib. One half was used for chlorophyll measurement and the second half for protein extraction. For chlorophyll extraction, 4 circular discs (10 mm in diameter) were taken, weighed and ground with quartz sand in 5 cm^3 of 80 % acetone. After filtration through *Whatman No. 1* paper, the extracts were centrifuged for 3 - 5 min and the resulting supernatant was used for chlorophyll determination by a UV-Vis spectrophotometer (*UV-2550*, *Shimadzu*, Tokyo, Japan) similarly as Mukherjee and Kumar (2007). Total proteins were extracted as described previously (Wang *et al.* 2003). Briefly, olive leaves were pulverized in liquid nitrogen in a mortar, washed extensively using tricholoroacetic acid-acetone, and then extracted using phenol-based method. Protein samples were used for electrophoresis and immunoblot analysis.

A polyclonal antibody raised against *Polygonum tinctorium* leaf β-glucosidase (Minami *et al.* 1996), reacted specifically with a 61 kD protein in the extracts for leaves 2 to 5, whereas the protein appeared to be barely detectable in leaf 1. The highest amount of β-glucosidase was present in leaf 5 (Fig. 1*A*). On the 2-D blot of leaf 5, the antibody detected three isoforms of β-glucosidase with isoelectric points (pI) 5.8 - 6.2, of which isoform 1 was the most abundant isoform, followed by isoform 3 (Fig. 1*B*). Similarly, three isoforms of β-glucosidase existed in the mesocarp of olive fruit (data not shown).

By using the software *Quantity one* (*Bio-Rad*,

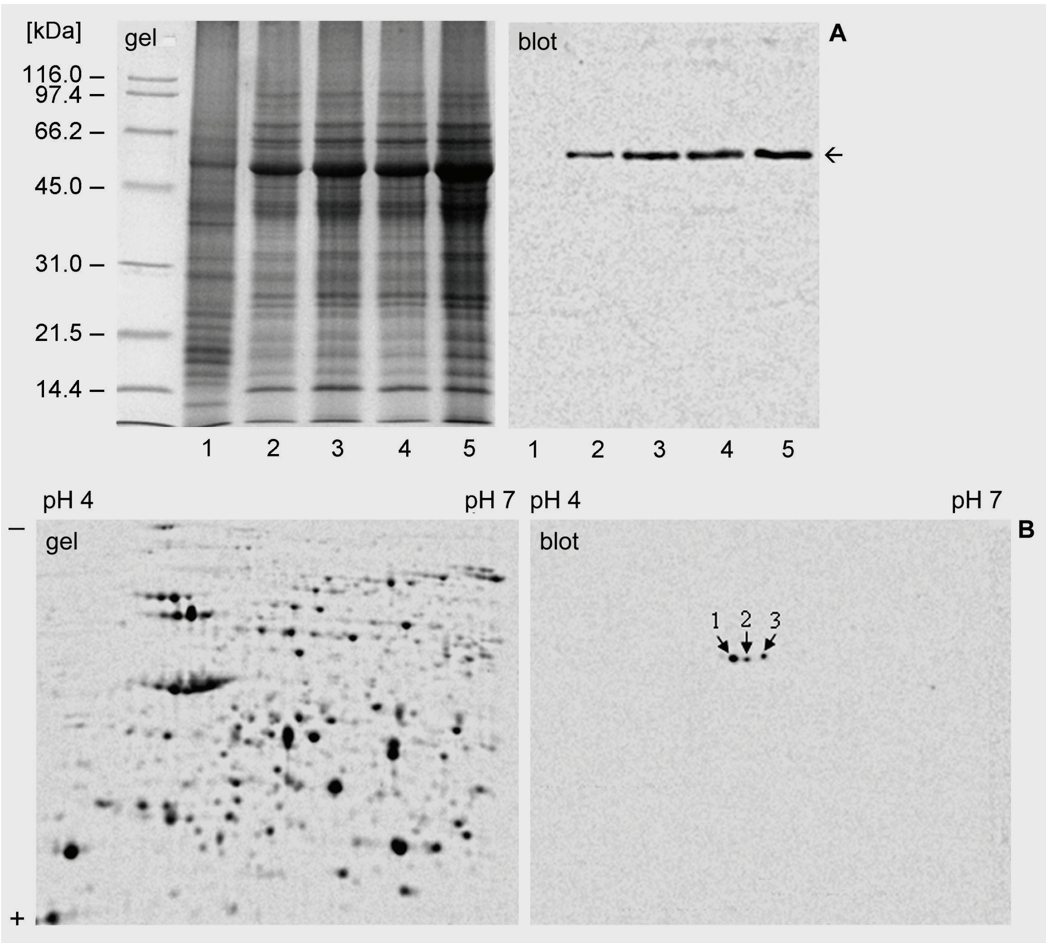

Fig. 1. Immunoblot analysis of β-glucosidase protein in olive leaves of different ages. This protein was resolved by electrophoresis, Coomassie blue stained or transferred to polyvinylidene difluoride membrane, and probed with anti-β-glucosidase antibody (1: 1000). The detection system is *ECL + Western Blotting Reagents* (*GE Healthcare*). Leaf position was numbered starting from the apex of a current-season branch, and leaf 1 was with a midrib length of 2.5 cm. *Lanes* 1 to 5 correspond to leaves No. 1 - 5, respectively. *A* - 12.5 % gel and blot. Each lane was loaded with 25 µg of protein. *Arrow* indicates the band of β-glucosidase (61 kD). The size of protein standards are indicated at the left. *B* - 2-D gel and blot. Protein (*ca.* 100 µg) was extracted from leaf 5, and first fractionated by isoelectric focusing (IEF) on 7 cm IPG strips (pH 4 - 7). IEF was conducted at 200 V for 1 min, at 300 V for 30 min, and finally at 3500 V for 2 h. The second SDS-PAGE was run in 12.5 % gel. *Arrows* indicate the isoforms of β-glucosidase.

Hercules, CA, USA), we determined the relative intensity of each β-glucosidase blot (Table 1). In general, the intensity increased with leaf age until the leaf fully was expanded. In other words, as leaf position increased in distance from the apex, increased amount of β-glucosidase was present per unit protein mass. Thus, the content of β-glucosidase protein was highly dependent on leaf age.

Table 1. Chlorophyll content and relative β-glucosidase content in olive leaves determined by using the software *Quantity one*. The correlation coefficient between chlorophyll content and relative β-glucosidase level was 0.93, computed with the *Correl* function in *Microsoft Excel*. Means ± SE (n = 2 - 3), * - values significantly different from the leaf 1 at 5 % level.

Leaf No.	Chlorophyll [g m^{-2}]	β-glucosidase [%]
1	0.68 ± 0.10	2.5
2	0.80 ± 0.12*	21.0
3	0.85 ± 0.11*	51.0
4	0.87 ± 0.12*	73.0
5	0.91 ± 0.11*	100.0

Chlorophyll content in olive leaves varied depending on the stage of leaf development (Table 1). Total chlorophyll content increased with leaf age for current-season leaves attached on the same branch. Maximum chlorophyll content in leaf 5 was 0.91 g m^{-2}. The content was similar with that previously reported (Pontikis and Hagidimitriou 2005). Statistical analysis indicated that there was a good correlation between chlorophyll content and the relative content of β-glucosidase (r = 0.93).

Previously, Minami *et al*. (1997) found that β-glucosidase expression is tissue specific in the leaves of indigo plants, and the enzyme was localized in the stroma of chloroplasts in mesophyll cells, but neither in the chloroplasts in marginal cells of vascular bunds and in epidermal cells. The subcellular localization study in olive fruit showed that β-glucosidase was mainly present in chloroplasts of mesocarp (Mazzuca *et al*. 2006). The developmental expression of β-glucosidase in olive leaves reported here is consistent with the intracellular localization of the enzyme.

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